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Exhibit E

Potent, long-acting bradykinin antagonists for a wide range of applications[1]

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Abstract: Actions of bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg; BK) are mediated by constitutively expressed B_2 receptors (which require the full BK peptide chain) and by B_1 receptors (which require BK(1–8) as ligand) that are induced in inflammation. BK has many functions in normal and pathological physiology, including initiation of most, if not all, inflammation. BK also evidently functions as an autocrine stimulant for growth of small cell lung cancer (SCLC). A new group of BK antagonists containing the novel amino acid α-(2-indanyl)glycine (Igl) provides both broad-spectrum and selective antagonists for all these functions. As examples, D-Arg-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic-Arg (B9430) is an extremely potent and long-acting antagonist of both B_1 and B_2 receptors, is stable against endogenous kininase enzymes, and is active in various in vivo models, including by intragastric administration. Acylation of B9430 with dehydroquinuclidine-2-carboxylic acid (Dhq) gives B9562, a highly selective B_2 antagonist. In contrast, Lys-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic (B9858) is a highly potent and selective B_1 antagonist. The dimer of B9430 linked at the amino terminus with suberimide is a potent selectively cytotoxic agent for SCLC cells. Results with these peptides suggest that a new generation of antiinflammatory and anticancer drugs may be at hand.

Key words: bradykinin, bradykinin antagonists, bradykinin receptors, cancer, inflammation, small cell carcinoma of lung.

Résumé : Les actions de la bradykinine (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg; BK) sont véhiculées par les récepteurs B_2 exprimés de manière constitutive (nécessitant la chaîne peptidique complète de la BK) et par les récepteurs B_1 (nécessitant BK(1–8) comme ligand) qui sont induits durant l'inflammation. La BK a de multiples fonctions en physiopathologie et en physiologie, notamment l'induction de la majeure partie, sinon de la totalité de l'inflammation. De plus, la BK fonctionne manifestement comme un stimulant autocrine pour le développement du cancer pulmonaire à petites cellules (CPPC). Dans un nouveau groupe d'antagonistes de la BK comportant le nouvel acide aminé α-(2-indanyl)glycine (Igl), on trouve tant des antagonistes à large spectre que des antagonistes sélectifs pour toutes ces fonctions. Par exemple, D-Arg-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic-Arg (B9430) est un antagoniste de longue durée et très puissant des récepteurs B_1 et B_2, qui est stable contre les enzymes kininases endogènes et actif dans divers modèles in vivo, notamment l'administration intragastrique. L'acylation de B9430 au moyen de l'acide déhydroquinuclidine-2-carboxylique (Dhq) donne B9562, un antagoniste B_2 très sélectif. À l'opposé, Lys-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic (B9858) est un antagoniste B_1 très puissant et très sélectif. Le dimère de B9430, lié à l'extrémité amino-terminale avec le suberimide, est un agent cytotoxique très sélectif pour les cellules CPPC. Les résultats obtenus avec ces peptides suggèrent qu'une nouvelle génération de médicaments anti-inflammatoires et anticancéreux pourrait être à notre portée.

Mots clés : bradykinine, antagonistes de la bradykinine, récepteurs de la bradykinine, cancer, inflammation, cancer pulmonaire à petites cellules.

[Traduit par la Rédaction]

Intr duction

The nonapeptide bradykinin (BK) and the homologous decapeptide kallidin (Lys-BK) are produced endogenously by enzymatic cleavage by plasma and tissue kallikreins of their circulating precursor proteins (kininogens) in many tissues and under a wide variety of conditions for regulation of both normal and abnormal physiology (Bhoola et al. 1992). In addition to their functions in regulation of normal physiology, there is considerable evidence to support the hypothesis that these peptides (collectively called kinins) are the initiators of most, if not all, inflammation (Stewart 1993). Trauma, infection, and allergic reactions have all been shown to stimulate kinin release; the kinins then stimulate release of the further chain of inflammatory mediators, such as prostaglandins, tumor necrosis factor (TNF), and various interleukins. A decade of animal studies and recent clinical trials have indicated that BK antagonists may become important drugs for antiinflammatory medicine.

Under normal conditions, actions of kinins are transitory in vivo as a result of rapid cleavage of the peptides by several enzymes. The most important of these are angiotensin-converting enzyme (ACE; kininase II), localized principally in

Received September 10, 1996.

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Table 1. Structures and receptor binding activities of BK and related peptides.

Peptide	Structure	Receptor binding[a] B_2	Receptor binding[a] B_1
BK[b]	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	8.9	<6
BK(1–8)[b]	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe	Inactive	5.6
Lys-BK(1–8)[b]	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe	4.7	9.0
Lys-[Leu[8]]BK(1–8)	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Leu	4.1	8.7
NPC-567	D-Arg-Arg-Pro-Hyp-Gly-Phe-Ser-D-Phe-Phe-Arg	8.2	6.4
HOE-140	D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg	9.8	6.0
B9340	D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Igl-Oic-Arg	9.8	8.1
B9430	D-Arg-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic-Arg	9.6	7.9
B9562	Dhq-D-Arg-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic-Arg	9.2	6.0
B9594	Aaa-D-Arg-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic-Arg	7.2	6.0
B9858	Lys-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic	7.7	10.1
B9870	SUIM -D-Arg-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic-Arg	8.4	7.9

Note: Aaa, adamantaneacetyl; Dhq, dehydroquinuclidine-3-carboxyl; Hyp, *trans*-4-hydroxyproline; Igl, α-(2-indanyl)glycine; Oic, octahydroindole-2-carboxylic acid; SUIM, suberimidyl; Thi, β-(2-thienyl)alanine; Tic, tetrahydroisoquinoline-3-carboxylic acid.

[a]Activities are given as the pEC_{50} for agonists and pIC_{50} for antagonists. Determined on IMR-90 human pulmonary fibroblasts (B_1) or cloned human B_2 receptors in CHO cells.

[b]Agonist.

the endothelium of the pulmonary vasculature, and the soluble circulating carboxypeptidase N (CPN; kininase I). The membrane-bound enkephalinase (endopeptidase 3.4.24.11) and aminopeptidase P (APP) are less active toward the kinins. ACE removes the C-terminal dipeptide from BK, yielding BK(1–7), which is inactive biologically. The products of CPN action, BK(1–8) and kallidin(1–9), while inactive at B_2 receptors, are the normal ligands for B_1 receptors. Products of cleavage by APP and endopeptidase 24.11 are totally inactive. ACE normally cleaves more than 99% of BK on a single passage through the pulmonary circulation, and CPN normally causes BK to have a plasma half-life of 15 s or less.

Biological actions of kinins are mediated by two classes of receptors: B_1 and B_2. Both classes of receptors have been cloned and sequenced from a variety of species. They are typical G protein coupled receptors having seven putative helical membrane-spanning segments. In various tissues, BK receptors are coupled to every known second messenger system. Prominent among these, and particularly important in inflammation, are phospholipase A_2 (PLA_2), with subsequent production of prostaglandins and leukotrienes, and phospholipase C (PLC), with subsequent stimulation of cell proliferation, for wound healing. B_2 receptors are constitutively expressed on the membranes of most cells and require the full chain of the kinin peptides, including the C-terminal arginine residue, for binding and activation. In contrast, B_1 receptors are not normally expressed in most tissues; their expression is stimulated in inflammation (Marceau 1995). Activation by kinins of vascular B_1 and B_2 receptors causes vasodilation and lowering of blood pressure. The severe fall in blood pressure (shock) of systemic bacterial infection appears to be initiated and sustained by production of BK. Bacterial enzymes produce BK, either by direct cleavage of circulating kininogens or by activation of kallikreins which then cleave kininogens (Maeda et al. 1996). The lipopolysaccharide (LPS) endotoxin of Gram-negative bacterial cell walls also stimulates production of BK and initiates release of TNF and lymphokines. A particularly vicious aspect of infection is that ACE is lost from the pulmonary circulation, causing kinins to be metabolized principally by CPN, thus producing large amounts of the ligands for the concomitantly induced B_1 receptors and causing shock.

Antagonists for BK B_2 receptors were introduced in 1984 (Vavrek and Stewart 1985) and stimulated a renaissance of kinin research. Rapid metabolism of kinins had made demonstration of physiological and pathophysiological roles for kinins very difficult. With tools available to block kinin receptors, demonstrations of participation of kinins in regulation of every major physiological system and initiation or mediation of much pathophysiology soon followed. The essential structural change in the BK molecule for production of antagonists was replacement of the 7-proline residue by a D-aromatic amino acid, most commonly D-Phe. This change yielded a weak partial antagonist. Additional changes to increase receptor affinity and decrease enzyme degradation yielded the useful "first-generation" B_2 antagonists (NPC-349; see Table 1). These antagonists had low affinity for BK receptors and showed short activity in vivo because of cleavage by CPN (Stewart and Vavrek 1991; Regoli et al. 1986).

Although B_1 antagonists had been described earlier (Regoli et al. 1977), they did not attract much interest until the demonstration that B_1 receptors, normally not present in most tissues, are expressed in chronic inflammation (Perkins et al. 1993; Marceau 1995). Replacement of the C-terminal phenylalanine in the normal B_1 ligands (BK(1–8) and kallidin(1–9)) by a hydrophobic aliphatic amino acid yielded the first B_1 antagonists. An example is [Leu[8]]BK(1–8). The first-generation B_1 antagonists, like the earliest B_2 antagonists, were rapidly degraded in vivo.

The "second generation" of B_2 antagonists was begun with introduction by Hoechst investigators of Icatibant (HOE-140)

(Hock et al. 1991) and followed by the Cortech Bradycor (CP-0127) (Cheronis et al. 1992) (see Table 1). In the first-generation B_2 antagonists, such as the Stewart NPC-349, although the D-amino acid residue at position 7 blocked action of ACE, and the N-terminal D-arginine residue blocked aminopeptidase action, these antagonists were still degraded by plasma CPN and by endopeptidase 24.11. Indeed, the first-generation B_2 antagonists, such as NPC-349, showed B_1 antagonist activity in vivo resulting from enzymatic removal of the C-terminal arginine (Regoli et al. 1986). The significant structural feature of Icatibant is the incorporation of imino acids, which greatly restrict peptide conformation and inhibit enzyme action, at positions 7 and 8. Incorporation of octahydroindolecarboxylic acid (Oic) at position 8 made this peptide resistant to cleavage by CPN and thus greatly extended its in vivo activity. The bulky D-tetrahydroisoquinolinecarboxylic acid (D-Tic) at position 7, combined with Oic^8, strongly restricts the conformational freedom of the important carboxyl end of the peptide to a shape evidently preferred by the B_2 receptors (Kyle et al. 1993). The very hydrophobic nature of these residues is probably also important, causing Icatibant to have a slow "on-time" and a very long persistence at or near receptors. Bradycor owes its increased potency to its dimeric nature, with perhaps some additional contribution from the hydrophobic character of the linker moiety. Despite these improvements, both of these antagonists are slowly degraded by plasma and tissue extracts. Endopeptidase 24.11 is probably important in this degradation.

Recently, a dramatic improvement (the "third generation" of BK antagonists) came with the introduction of α-(2-indanyl)-glycine (Igl) into the antagonist structure (Stewart et al. 1996). An extremely interesting peptide is B9430, which has L-Igl at position 5, D-Igl at position 7, and Oic at position 8. This antagonist shows truly impressive high potency and long duration of action in vivo. The Igl residue at position 5 evidently blocks degradation by endopeptidase 24.11. These new antagonists persist more than 6 h in plasma and tissue homogenates and show very long duration of action in vivo. A single intravenous injection of B9430 in rats blocks the hypotensive action of BK for more than 4 h, and a subcutaneous injection in rabbits blocks BK action for more than 24 h. Perhaps the most remarkable property of the antagonists containing Igl is their high potency at B_1 receptors, in addition to the anticipated B_2 activity, although they contain the C-terminal arginine residue that normally prevents B_1 receptor activity of agonists and antagonists. Activity of these new antagonists at both receptors has been demonstrated in cultured cells, in isolated smooth muscle tissues, and in vivo. They are active at human B_1 and B_2 receptors. Most recently, B9430 has been shown to be active following intragastric administration in rats, although the bioavailability is low (Whalley et al. 1997). This result suggests that we may have made progress on the way toward the ambitious goal of a chemically modified peptide having significant oral activity.

BK has important growth factor activity, although the lability of BK has made demonstration of this property difficult. Production of BK in trauma (BK is produced whenever blood clotting is initiated) is probably to stimulate wound repair, where it can act in concert with platelet-derived growth factor. Recent papers have begun to delineate the intracellular events, especially tyrosine phosphorylation, that follow action of BK on cells (Tallett et al. 1996). Small cell lung cancer (SCLC) cells express BK receptors, and evidently use BK and other peptides (substance P, bombesin) as growth stimulants (Woll and Rozengurt 1988). Several peptide antagonists have been tested as potential inhibitors of SCLC growth, and some progress has been reported (Staley et al. 1991). Our BK antagonists have been tested consistently by D. Chan at the University of Colorado Cancer Center for their effects on cultured cells of SCLC. Whereas all our good antagonists, especially the new third-generation peptides, block the BK-evoked increase in intracellular calcium concentration (Bunn et al. 1994), they do not inhibit cell growth. Most recently, however, dimers of our new antagonists, such as B9870, were found to inhibit growth of cultured SCLC cells (Chan et al. 1996).

Receptor selectivity of peptide BK antagonists

As mentioned above, the presence or absence of the carboxy-terminal arginine is the usual determinant for receptor selectivity of kinin agonists and antagonists. It was therefore surprising to find that our full-chain antagonists containing a D-Igl residue at position 7 are good antagonists at B_1 receptors, in addition to the anticipated B_2 receptor antagonism. Icatibant, which has D-Tic at position 7, is without functional B_1 antagonist activity, although it does show some low binding to human ileum B_1 receptors. With the single change to D-Igl at position 7 (antagonist B9340), good functional B_1 antagonist activity is obtained in all systems examined, and the binding potency on human ileum B_1 receptors is increased by two orders of magnitude over that of Icatibant. At the same time, B_2 antagonist binding and functional activity are excellent.

The actual modes of binding of kinins and their antagonists to B_1 and B_2 receptors are not known. Site-directed mutagenesis studies of B_2 receptors (Freedman and Jarnagin 1992; Nardone and Hogan 1994; Novotny et al. 1994; Jarnagin et al. 1996) have indicated receptor residues that appear to be involved in binding of peptides to the receptor and have shown that different residues are involved in binding of BK and Icatibant (Herzig and Leeb-Lundberg 1995; Nardone and Hogan 1994). Based on these studies, Kyle et al. (1994) and Jarnagin et al. (1996) have proposed models for interaction of BK with the B_2 receptor. The amino acid sequence of the B_1 receptor was determined only recently, and mutagenesis studies on it have not yet been reported. These receptor studies have not offered any explanation for the ability of $D\text{-}Igl^7$ antagonists containing the C-terminal arginine to interact effectively with B_1 receptors. Examination of the structures of Igl and Tic shows that whereas both are large hydrophobic, aromatic residues of nearly the same size, Igl gives more flexibility at position 7, both to the side chain and to the peptide backbone. Yet the original B_2 antagonists with D-phenylalanine at position 7 showed no antagonism at B_1 receptors, and D-phenylglycine and D-homophenylalanine at position 7 did not yield antagonists. Analogs containing the L-isomers of Tic and Igl at position 7 are agonists.

Although the new [Igl^5,D-Igl^7]antagonists provide high potency at both B_1 and B_2 receptors, the structures of these remarkable peptides can be manipulated to provide highly selective B_1 or B_2 antagonists. Although the carboxy-terminus of BK and its agonist and antagonist analogs allows few

changes with retention of high potency, it has long been known that the N-terminus of BK and related peptides can be modified in many ways, particularly addition of amino acids or acyl groups, with retention of high potency. One early modification was acylation of first-generation antagonists with large, hydrophobic acyl groups such as adamantaneacetyl- or adamantanecarboxyl-; this was reported to increase potency (Lammek et al. 1991). Application of this modification to our new antagonists containing Igl (such as B9340 and B9430) gave the surprising result that whereas B_2 potency might be increased, B_1 potency was severely decreased. Thus, B9594, the adamantaneacetyl derivative of B9430, is a very potent, highly selective B_2 antagonist (three orders of magnitude more potent at B_2 than at B_1) with longer persistence of action in vivo than Icatibant. Additional exploration of N-terminal modifications of these new antagonists confirmed the observation seen with B9594 that high basicity at the N-terminus is essential for high B_1 antagonist potency. Applying this principle to [des-Arg^9]analogs, B9858, having an additional lysine residue at the N-terminus, is a potent antagonist having three orders of magnitude selectivity for B_1 over B_2 receptors, again retaining the long duration of in vivo action characteristic of the peptides containing Igl at positions 5 and 7 of the BK structure.

Applications for BK antagonists

The availability of the new enzyme-resistant BK antagonists opens new areas for experimental investigation and for new potential clinical applications. Now amenable to study are in vivo applications where an effective level of BK antagonist must be maintained constantly; this can now be done with once-daily injections. The expense of Alzet minipumps for such experiments can thus be avoided. Of particular interest are models of chronic inflammation. Since BK B_1 receptors are involved in the later stages of inflammation, selective B_2 antagonists are not efficacious in late stages. Use of peptides such as B9430, having both B_1 and B_2 antagonist activity, makes treatment with a single compound possible. B9430 has been shown to inhibit hyperalgesia and edema in both the early and late stages of carragenan-evoked inflammation in the rat footpad model (Stewart et al. 1996).

Following favorable animal results, the Cortech B_2 antagonist Bradycor was found to be effective in decreasing intracranial pressure in human closed head trauma (Rodell 1996). By the second day following such trauma, a full complement of B_1 receptors should be present, and use of a combined B_2–B_1 antagonist seems to be indicated. Severe spinal injury is another case where potent BK B_1–B_2 antagonists should be extremely useful. It is generally recognized that the poor outcome of spinal trauma is to a large degree the result of inflammation-induced tissue degradation following the injury. All evidence suggests that BK is a major active agent in this inflammation, and both B_1 and B_2 receptors are presumably involved. B9430 should be ideal for these types of applications.

Appli ati n f BK antag nist t lung anc r

The inflammatory response stimulated by tumors is probably an important factor in their invasive nature. This may be the basis for the recent discovery that low-dose aspirin (which inhibits prostaglandin production) causes a dramatic inhibition of incidence of colon cancer. Because BK is a major stimulus of PLA_2, which releases arachidonic acid for prostaglandin production, and because BK and prostaglandins act synergistically, especially in pain production, it is logical to investigate BK antagonists as potential therapeutic agents for cancers. Expression of several peptide receptors on cells of SCLC has led to the assumption that such peptides having growth factor activity are involved in the rapid growth and progression of SCLC. Receptors for gastrin-releasing hormone (bombesin), substance P, and BK are commonly found on SCLC cells; the most frequently occurring of these is the BK B_2 receptor.

The initial event triggered by binding of a growth factor to its receptor is an increase in intracellular free calcium concentration. SCLC cells show this response when challenged with a peptide growth factor for which they express receptors. Most BK B_2 antagonists inhibit the BK-evoked rise in calcium in these cells, but do not inhibit growth, whereas dimers of certain of our new antagonists containing Igl do inhibit growth and cause cell death. Cytotoxic potency depends on the peptide sequence, the nature and length of the linker joining the two peptides, and the position of attachment of the linker to the peptide. In studies so far, the structure shown in Table 1 (B9870) is among the most potent and best studied. B9870 consists of two molecules of B9430 linked at the N-terminus with the eight-carbon suberimide group. The more basic nature of the suberimide linker, compared with the neutral suberyl linker, maintains B_1 antagonist activity in the dimer. Preliminary evidence suggests that the mechanism of killing is induction of apoptosis. It is remarkable that killing is equally effective in SCLC lines that have developed multiple drug resistance toward standard chemotherapeutic agents. Preliminary results from a study of B9870 in vivo in nude mice implanted subcutaneously with SCLC show significant inhibition of tumor growth.

Peptides versus nonpeptides as BK antagonists

It is a widely held dogma in the pharmaceutical industry that successful drugs, especially for chronic conditions, must be orally active. Given this attitude, one might dismiss the effort to develop better peptide BK antagonists as a mere academic exercise without practical significance and await the development of suitable nonpeptide antagonists. Much evidence, however, suggests that this may not be the wisest course of action.

Until now the only BK nonpeptide antagonist described is that of the Sterling–Winthrop group (Salvino et al. 1993). This antagonist, WIN-64338, shows high potency at some receptors, including human B_2 receptors, and is orally available, but it is not highly selective for BK receptors. At somewhat higher concentrations, it also blocks acetylcholine muscarinic receptors. In retrospect, this activity at acetylcholine receptors may not be too surprising because WIN-64338 contains two basic groups separated by a distance similar to that separating the basic charges in hexamethonium chloride, a standard, potent antagonist for acetylcholine receptors. Given this lack of selectivity, it is remarkable that the WIN antagonist is inactive at rat uterus BK receptors (J.M. Stewart, unpublished observation). Rat uterus B_2 receptors were the first to be cloned and sequenced and are considered to be typical B_2 receptors. This

puzzling lack of activity in the uterus, along with the cross-reactivity with muscarinic receptors, is illustrative of the kinds of unexpected problems that can arise when developing non-peptide mimics of peptide drugs.

This is not to imply, however, that problems with the WIN antagonist may not be satisfactorily overcome in new antagonists. One such, developed at Fujisawa, in Japan, is being described at this symposium (Inamura et al. 1997). Results of studies with this and other new compounds are awaited with interest.

However, peptides should not yet be dismissed. Orally effective peptide drugs may be developed. Indeed, our antagonist B9430 is orally active in the rat, although bioavailability is low (Whalley et al. 1997). Buccal or intranasal routes of administration should be effective for peptides of this size range. Emergency medicine needs parenterally active drugs for production of immediate results, for example, in severe trauma and septic shock; peptide BK antagonists offer tools for these needs. Cortech's Bradycor has been shown to be marginally effective in certain forms of septic shock, is effective in treatment of human closed head trauma (Rodell 1996), and is in continuing clinical trials. There is every reason to believe that BK antagonists may provide badly needed drugs for spinal injury; paraplegia and quadriplegia are more often the result of posttrauma inflammation, rather than of actual damage to the spinal cord. Icatibant relieves nasal blockade in patients with seasonal allergic rhinitis (Austin et al. 1994) and is effective in certain forms of asthma (Akbary et al. 1996). Our Igl-containing antagonists show much better properties than these antagonists and probably are ready for clinical development.

In favor of peptide BK antagonists for immediate development for clinical uses is the fact that several peptide BK antagonists have been used in humans without evidence of any harmful side effects. The problems with the antagonists available until now have been lack of potency and duration of action, but these problems have now been overcome. The long experience with peptides has enabled us to produce antagonists with precisely defined effects — highly specific or broadly efficacious, but still restricted in action to kinin systems. Thus, peptide antagonists are ready for immediate development for treatment of life-threatening conditions, with the promise of continued usefulness with hospitalized patients, even beyond the time when nonpeptide BK antagonists may be developed.

Nonpeptides do unquestionably offer the potential for development of more suitable drugs for chronic inflammatory conditions. Because of the established synergism between BK and prostaglandins in production of pain and inflammation, such BK antagonists may in time become useful adjuncts to cyclooxygenase inhibitors (glucocorticoids and nonsteroidal antiinflammatory agents). Relative cost of production of peptide versus nonpeptide drugs is also an important factor, but must of necessity be evaluated for each drug pair.

Acknowledgments

Research in the Stewart laboratory was supported by National Institutes of Health grant HL-26284. The authors thank Paul Bury for assistance with chemistry and Frances Shepperdson for bioassays.

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